

1 PREVENTION OF PRIMARY SJÖGREN'S SYNDROME BY ICA69 DEFICIENCY

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3 CROSS-REFERENCE TO RELATED APPLICATIONS

4 This application relies upon U. S. Provisional Patent
5 Application No. 60/415,879, filed October 3, 2002, the
6 contents of which are herein incorporated by reference in its
7 entirety.

8

9 FIELD OF THE INVENTION

10 This invention relates to identification of an
11 autoantigen implicated in the development and progression of
12 primary Sjögren's Syndrome (pSS); particularly to the disease
13 modifying effect of creating a deficiency in the ICA69
14 autoantigen; and most particularly to development of
15 diagnostic and therapeutic avenues, means for the
16 differential diagnosis of pSS versus other autoimmune
17 disease, e.g. Systemic lupus erythematosis (SLE), and
18 procedures for immunotherapeutic treatment effective to alter
19 the course and progression of pSS.

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1 BACKGROUND OF THE INVENTION

2 Primary Sjögren's Syndrome (pSS) is a common, chronic
3 autoimmune disorder of unknown etiology, affecting exocrine
4 glands, primarily (90%) in middle-aged women with a
5 prevalence varying between 0.3-4.8%, depending on region and
6 diagnostic criteria.

7 Despite considerable efforts to find evidence of an
8 initiating viral trigger, the cause of Sjögren's Syndrome
9 remains unknown. The disease leads to lacrimal and salivary
10 dysfunction, with dryness of mouth and eyes leading to
11 considerable surface damage and attendant chronic discomfort
12 and pain. The disease involves activation of CD4-predominant
13 T cells and of B lymphocytes with autoantibodies detectable
14 in the circulation, and associated with complications such as
15 vasculitis and interstitial pneumonitis. The chronic B cell
16 activation can lead to the slow emergence of autonomous
17 clones of B cells that can evolve into non-Hodgkin's lymphoma
18 at a rate that is 44 times that of the general population (an
19 incidence around 6.5%). There is growing evidence that a
20 subset of patients may have or develop multiple sclerosis.
21 Liver disease such as Primary Biliary Cirrhosis and
22 Autoimmune Hepatitis can be associated with Sjögren's
23 Syndrome.

1 Pathologically, the hallmark of pSS is a CD4-predominant
2 glandular T cell infiltrate that is initially periductal, and
3 later leads to B cell and plasma cell accumulation. The
4 secretory defect occurs disproportionately to the degree of
5 acinar destruction, such that the early dryness is thought to
6 result from immunological targeting of the muscarinic 3
7 parasympathetic receptors within the glands. Infiltrates in
8 salivary and/or lacrimal glands, eventually lead to tissue
9 destruction, and this is thought to occur in part because of
10 targeting of a number of autoantigens, such as alpha and beta
11 fodrin, and protein fragments associated with intracellular
12 RNA, such as Ro and La. The original observation of the
13 instant inventors of strong protection from salivary, and
14 complete absence of lacrimal disease in ICA69-deficient NOD
15 mice was unexpected, as previous work associated this
16 autoantigen specifically with human and NOD type 1 diabetes,
17 and, more recently, multiple sclerosis. ICA69 is a self-
18 antigen expressed in brain, pancreas, salivary and lacrimal
19 glands. NOD-strain mice represent a premier animal model of
20 spontaneous pSS.

21 Organ-selective autoimmune disorders are characterized
22 by broad spreading to multiple target autoantigens, and the
23 genetic removal of any one such antigen was expectedly not
24 associated with significant disease impact in autoantigen
25 gene knockouts (GAD65, ICA69, IA2), the recent observation of
26 T1D protection in insulin-1 knockouts raised questions of the

1 degree of backcrossing, since heterozygous animals also show
2 protection. Reduced antigen spreading may set Sjögren's
3 Syndrome apart, perhaps due to lesser involvement of CD8+ T
4 cells that drive disease progression in conditions such as
5 autoimmune diabetes.

6 The clinical picture varies and can be stable or
7 progressive, occasionally leading to life threatening
8 complications. Therapeutic approaches in pSS are symptomatic
9 and, on the whole, considered inadequate. It is often
10 difficult to justify the routine use of immunosuppressive
11 drugs because the disease is so localized, and the downside
12 of these medications would seem to be excessive, in
13 particular considering the possible risk of accelerating
14 lymphoma and increased risk of infection. As in other
15 autoimmune disorders, most immunosuppressants tested have
16 shown limited effectiveness in Sjögren's Syndrome. Thus pSS
17 is a prototypical, tissue-selective autoimmune disorder, and
18 it shares many fundamental aspects with its cousins, MS, type
19 1 diabetes, Crohn's disease and others.

20 Animals can develop homologs of Sjögren's Syndrome. The
21 premier pSS model, NOD-strain mice, provide the closest
22 approximation of the human disease. NOD pSS develops
23 independently of type 1 diabetes, and does not require the
24 diabetes-prerequisite NOD MHC class II (I-Ag⁷). We have
25 generated knockout mice, deficient in the diabetes
26 autoantigen, ICA69, and bred the null allele onto NOD

1 congenic animals. While Type 1 diabetes (T1D) development
2 proceeded at slower rate but normal incidence, these mice
3 showed a dramatic reduction of pSS, with complete prevention
4 of the lacrimal disease typical for old males.

5 In wild-type NOD mice, immunotherapeutic induction of
6 tolerance to ICA69 has been optimized and is effective at
7 reversing sialoadenitis and dacryadenitis even in late stage
8 disease.

9 Autoimmunity in, for example, Type 1 diabetes, is
10 characterized by progressive spreading to many different
11 autoantigens, and to more epitopes within each. The inability
12 of ICA69 deficiency (or for that matter, GAD65 or IA2
13 deficiency) to affect T1D outcome was therefore not
14 surprising. This, then, sets pSS apart, and suggests that
15 autoimmunity in this disease is considerably more narrow with
16 less antigen spreading, perhaps consistent with the
17 surprising effectiveness of ABBOS immunotherapy. pSS
18 protection was complete only for lacrimal disease, but there
19 was low grade, and less progressive salivary disease in the
20 KO mice, suggesting that the process underlying and driving
21 the autoimmune attack was still at work, presumably targeting
22 otherwise perhaps minor target autoantigens.

23 Initially, ABBOS mediated pSS protection was not quite
24 uniform, and a subset of treated animals showed little
25 protection, a few even disease acceleration. This was not
26 surprising, and likely dose related, since previous work had

1 demonstrated that a suboptimal ABBOS dose can mimic the
2 effect of Tep69 and precipitate disease. These observations
3 were initially made in animals receiving single injections,
4 however treatment protocols have now been optimized, and the
5 instantly disclosed protocol shows no acceleration.

6 In a small study of pSS patients, nearly all had
7 prominent T cell autoreactivity to ICA69, that targeted the
8 same epitope as the immunodominant target typical for T1D.

9 As a necessary prelude to phase I immunotherapy trials,
10 it is now proposed to use NOD mice to further optimize pSS
11 immunotherapy for subsequent translation to the human system,
12 extend studies of ICA69 autoimmunity in pSS patients (and
13 their relatives), establish MHC immunogenetics of these T
14 cell responses, systematically map human pSS epitopes and
15 conduct T cell mechanistic studies.

16 These studies are expected to form a rational basis for
17 tolerance-inducing peptide infusions alone or in combination
18 with other disease modifying drugs in pSS patients. Since the
19 Syndrome is largely localized to salivary and lacrimal
20 glands, direct tissue access and secretory function measures
21 are possible, and indeed have been used to assist in the
22 routine diagnosis of pSS. This disease thus appears to be a
23 prime candidate to become the test- and development platform
24 for immunotherapy of organ-selective autoimmune diseases in
25 general, which has so far failed to translate broadly
26 encouraging rodent data to humans.

1 Glossary of Terms:

2 ABBOS T cell epitope in bovine serum albumin (BSA).

3 IFA incomplete Freund's adjuvant (water-oil emulsion).

4 MHC major histocompatibility complex, e.g. HLA in
5 humans, H-2 in mice.

6 Mimicry antigenic cross-reactivity: e.g. Tep69 & ABBOS
7 peptides are recognized by the same T cell clones and auto-
8 antibodies.

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11 NOD non-obese diabetic mice, develop primary
12 Sjögren's Syndrome spontaneously and independently of Type 1
13 diabetes.

14 Tep69 T cell self-epitope in ICA69.

15

16 DESCRIPTION OF THE PRIOR ART

17 U.S. Patent No. 6,207,389 is directed toward methods of
18 controlling T lymphocyte mediated immune responses and to
19 methods of detecting subjects at risk for developing Type I
20 Diabetes by detection of antibodies to p69 protein.

21

22 SUMMARY OF THE INVENTION

23 In accordance with the present invention the genomic
24 ICA69 locus was inactivated, thereby generating ICA69-
25 deficient NOD congenic mice which were subsequently analyzed

1 for the development of pSS. ICA69 autoimmunity was analyzed
2 in controls or patients with primary SS or SLE, and in
3 various NOD mice, some treated with an ICA69-directed
4 prototype peptide vaccine.

5 Disruption of the ICA69 locus was found to prevent
6 lacrimal and dramatically reduced salivary gland disease in NOD
7 mice. In normal NOD mice, ICA69-specific T-cells accumulated in
8 lymph nodes draining salivary tissue. Patients with primary SS,
9 but not SLE patients, nor healthy control subjects, had similar
10 T- and B-cell autoreactivity against ICA69. Immunotherapy with
11 a high-affinity mimicry-peptide targeting ICA69-specific T-
12 cells produced long-term reduction of established pSS in wild
13 type NOD mice.

14 ICA69 is a new autoantigen in primary SS that plays a
15 critical role in disease progression and may be of diagnostic
16 value. Immunotherapy of primary SS with a high-affinity
17 mimicry-peptide targeting ICA69-specific T-cells appears to be
18 promising, since autoimmunity in NOD pSS appears uniquely
19 susceptible to such treatment even late in disease.

20 Accordingly, it is an objective of the instant invention
21 to identify an autoantigen implicated in the development and
22 progression of Sjögren's Syndrome (pSS).

23 It is a further objective of the instant invention to
24 demonstrate the disease modifying effect of creating a
25 deficiency in the ICA69 autoantigen.

26 It is yet another objective of the instant invention to

1 develop diagnostic and therapeutic avenues for treatment of
2 pSS.

3 It is a still further objective of the invention to
4 provide means, e.g. diagnostic assays, for the differential
5 diagnosis of pSS versus other autoimmune disease, e.g.
6 Systemic Lupus Erythematosis (SLE).

7 It is yet an additional objective of the invention to
8 develop procedures for immunotherapeutic treatment effective
9 to alter the course and progression of pSS.

10 It is a still further objective of the instant invention
11 to teach a transgenic animal, particularly an ICA69 deficient
12 NOD mouse, which essentially does not develop pSS.

13 Other objects and advantages of this invention will
14 become apparent from the following description taken in
15 conjunction with the accompanying drawings wherein are set
16 forth, by way of illustration and example, certain
17 embodiments of this invention. The drawings constitute a
18 part of this specification and include exemplary embodiments
19 of the present invention and illustrate various objects and
20 features thereof.

21

22 BRIEF DESCRIPTION OF THE FIGURES

23 The instant patent or application file contains at least
24 one drawing executed in color. Copies of the patent or
25 patent application publication with color drawing(s) will be
26 provided by the Office upon request and payment of the

1 necessary fee.

2 **Figure 1.** Protection from sialoadenitis and absence of
3 dacryoadenitis in ICA69 deficient NOD mice.

4 **Figure 2.** Measurement of T cell proliferative responses to
5 ICA69, its dominant epitope, Tep69, BSA, and its dominant NOD
6 mouse epitope, ABBOS, measured in lymph nodes draining the
7 pancreas.

8 **Figure 3.** Modification of sialoadenitis by peptide-based
9 immunotherapy.

10 **Figure 4.** Splenic T cell responses to Tep69 in ABBOS-treated
11 mice with persistent sialoadenitis (n=6, green or blue
12 shading in A), and mice with peptide-mediated disease
13 reduction (n=11, red shading in A).

14 **Figure 5.** Pilot studies were used to hone in on 3 variables:
15 peptide dose, route of administration (i.v., i.p., s.c.)
16 which effect the success of pSS immunotherapy.

17 **Figure 6.** Illustration of effectiveness of ABBOS peptide-
18 based vaccine, and involvement of anti-mAChR autoantibodies
19 in affecting salivation.

20 **Figure 7.** T and B cell autoimmunity to ICA69 in patients with
21 primary SS, and SLE versus healthy controls.

22 **Figure 8.** T cell and B cell autoimmunity to ICA69 in
23 patients with pSS.

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1 DETAILED DESCRIPTION OF THE INVENTION

2 Discussion of Figures

3

4 **Figure 1.** Protection from sialoadenitis and absence of
5 dacryoadenitis in ICA69 deficient NOD mice. (A) Female
6 ICA69⁺⁻ and ICA69^{-/-} NOD mice were sacrificed at various ages
7 and the number of mononuclear cell foci in both submandibular
8 glands were enumerated. *P > 0.1; **P < 0.01; ***P < 0.001.
9 (B) Representative histopathology of submandibular glands
10 from ICA69⁺⁻ and ICA69^{-/-} NOD mice of various ages (H&E
11 stains, 40X magnification). (C) Histological signs of
12 dacryoadenitis, observed in most ICA69⁺⁻ NOD males, is absent
13 in ICA69^{-/-} NOD males aged 35-40 weeks (H&E stains, 100X
14 magnification).

15 **Figure 2.** T cell proliferative responses to ICA69, its
16 dominant epitope, Tep69, BSA, and its dominant NOD mouse
17 epitope, ABBOS, were measured in lymph nodes draining the
18 pancreas (A), and submandibular glands (B), or lymph nodes
19 draining the lower (C) or upper (D) extremities. Gray
20 columns: control cultures stimulated with ovalbumin (OVA) or
21 Medium (MED) only. To obtain sufficient cell numbers, lymph
22 node cells were pooled from seven mice. One of three similar
23 data sets is shown.

24 **Figure 3. Modification** of sialoadenitis by peptide-based

1 immunotherapy. (A) 10 week-old NOD females received 200 µg
2 ABBOS i.p. in incomplete Friend's adjuvant (IFA), vehicle
3 only (PBS) or were left untreated. Sialoadenitis scores were
4 measured 5, 10 or 15 weeks later. Colour key: protected mice
5 - red, unchanged sialitis - green, enhanced disease - blue.
6 (B) Submandibular gland from a 20 wk old NOD female
7 previously injected with PBS-IFA. Absence (C), reduction (D),
8 or increase (E), of sialoadenitis in submandibular glands
9 from 20 wk old NOD females injected with ABBOS peptide 10
10 weeks earlier (H&E stains, 40X magnification).

11

12 **Figure 4.** Mechanisms of immunotherapy-induced disease
13 protection are at best partially resolved in general. In
14 terms of T cell autoreactivity in the present context, **only**
15 protected animals showed an absence (fat arrow vs. thin
16 arrow) of T cell pools that recognized both, ABBOS and its
17 endogenous ICA69 mimicry peptide, Tep69 (see figure). The
18 instant inventors have constructed ICA69 transgenic NOD mice
19 which showed deviation of mimicry T cells recognizing the
20 Tep69 epitope as well as ABBOS: these mice were protected
21 from autoimmune disease, and formally demonstrated the
22 protective abilities of ABBOS-only T cell pools noted earlier
23 in functional studies in NOD mice and humans. The explanation
24 for these observations is, almost certainly, that deviation

1 of the fine specificity of T cell receptors for Tep69 is
2 associated with loss of pathogenicity in the remaining T cell
3 pools. However, it remains possible that lasting T cell
4 anergy might play a role in disease protection and/or the
5 undetectability of relevant (Tep/ABBOS-specific) T cell
6 pools.

7

8

9 **Figure 5.** Pilot studies were used to hone in on 3 variables:
10 peptide dose, route of administration (i.v., i.p., s.c.) and
11 injection schedules (the published data (*Lancet*) used single
12 injection, 100 μ g, s.c. in IFA (i.e. oil), the latter would
13 not likely be usable in humans and we now have tested i.p.
14 without IFA). We also refined our sialitis scoring system to
15 include a correction for gland weight: this strategy reduced
16 variability even of previous data considerably, enhancing our
17 statistical power. To our surprise, i.v. injection (effective
18 at T1D prevention) failed to affect pSS. Pilot studies also
19 suggested that 3 injections 2 weeks apart were more effective
20 than a single large injection, and this schedule prevented
21 unsuccessful as well as accelerating outcomes.
22 The new data shown in the figure derive from a large,
23 complete experiment to test these pilot suggestions. "Large
24 infiltration foci" are the main pathogenic infiltrates

1 associated with tissue destruction and disease progression.
2 Injection of ABBOS, 5 mg/kg (roughly equivalent to
3 100 μ g/mouse) turned out to be an effective dose, 3-10 times
4 larger doses were not more effective. We chose to begin
5 therapy at an age of 10-12 weeks, when salivary disease in
6 females is well established with high incidence (around 85%
7 in our colony and at least mild disease in most animals). Two
8 further injections followed, 3 weeks apart. As shown in the
9 figure, s.c. injection was the superior route ($p<0.0001$ vs.
10 ABBOS i.p., PBS or OVA peptide injection). Salivary data were
11 obtained in females at 25-30 weeks or earlier if animals
12 developed T1D (red symbols).

13

14 **Figure 6.** The plausible involvement of anti-mAChR
15 autoantibodies in affecting salivation, requires
16 consideration. The ABBOS peptide-based 'vaccine' was
17 effective in NOD pSS, as judged by pathohistology (scoring of
18 infiltrative foci) and data on recovery of secretory function
19 (Figure, $p=0.002$ ABBOS vs. control treatments). However, no
20 data were obtained on mAChR autoantibodies. Given the rather
21 short Ig-half life times in mice, and the almost certain T
22 helper cell dependency of such antibodies, it is possible
23 that T cell directed immunotherapy will reduce autoantibody
24 levels, and secretory function. Normalized exocrine secretion

1 may imply that successful immunotherapy does affect
2 autoantibodies that interfere with secretion.

3 **Figure 7.** (A) T cell responses to ICA69, BSA, Tep69, and ABBOS
4 were analyzed in patients with primary SS (n=9) SLE patients
5 (n=6) or healthy controls (n=12). Positive responses to
6 tetanus toxoid (TT) contrasted with negative responses to
7 OVA, actin or the type 1 diabetes-associated GAD65 peptide,
8 p555. Data are expressed as stimulation index (SI,
9 experimental/background cpm, as described herein). Background
10 counts were similar in all cohorts (mean \pm SD: 1154 \pm 354 cpm).

11

12 **Figure 8.** Autoantibodies to ICA69 (1 μ g protein/lane) were
13 detected in Western blots of sera (1 to 1000 dilution) from
14 patients with pSS (lanes 1-5) but not in controls (lanes 6-8).

15

16 pSS is a chronic autoimmune disease characterized by
17 lymphocytic infiltration and destruction of exocrine glands,
18 in particular in salivary and lacrimal tissue¹. Destruction
19 of these glands often results in dryness of the eyes
20 (keratoconjunctivitis sicca), and mouth (xerostomia). The
21 prevalence of the disease is high, with about 1% of the
22 population affected, most being females. Both organ selective
23 and systemic autoimmunity are thought to participate in
24 disease progression. As with other organ-selective autoimmune

1 disorders, there is evidence for multiple environmental and
2 genetic factors that contribute to disease risk in pSS^{2,3}.
3 Several candidate autoantigens associated with pSS have been
4 identified and some are currently used in disease diagnosis.
5 Of these, SS-A/Ro, SS-B/La, and the recently identified SS-56
6 are considered systemic autoantigens and have been linked to
7 other autoimmune diseases such as systemic lupus
8 erythematosus (SLE)^{4,5}. In addition, autoantigens such as a-
9 fodrin, b-fodrin, and the muscarinic M3 receptor are
10 considered tissue-restricted autoantigens in pSS⁶⁻⁸. The
11 pathogenic roles of these autoantigens in the initiation and
12 progression of pSS are unclear, but antibodies against the
13 muscarinic M3 receptor may participate in the loss of
14 salivary function⁸. pSS treatment is essentially symptomatic.
15 Identification of new autoantigens and their pathogenic roles
16 could have considerable impact on design of new diagnostic
17 and therapeutic strategies¹.

18 Several animal models have been used to study pSS,
19 including the nonobese diabetic (NOD) mouse, the MRL/lpr
20 mouse and the NFS/sld mouse, thymectomized 3 days after
21 birth⁹⁻¹¹. Among these, the NOD mouse may represent the
22 premier model, since, like in human pSS, loss of salivary
23 secretory function develops spontaneously^{8,12}. The NOD mouse
24 is also the premier model for spontaneous type 1 diabetes,

1 but the two diseases can be separated genetically; for
2 example, NOD.H-2^b mice develop pSS, but not diabetes¹³.
3 NOD mice, like human diabetes patients and many relatives
4 with a high genetic risk to develop diabetes, lose tolerance
5 to the islet cell autoantigen 69 kDa, ICA69^{14,15}. ICA69 is a
6 conserved protein of unknown function whose expression
7 pattern includes neurons, pancreatic b-cells, salivary and
8 lacrimal glands¹⁶⁻¹⁸. T- and B lymphocytes from NOD mice and
9 the majority of diabetes patients target primarily the ICA69-
10 36 epitope, Tep69, although other cryptic epitopes likely
11 exist^{14,15}. ICA69 (but not its Tep69 epitope) is also targeted
12 in multiple sclerosis¹⁹. We recently generated speed-congenic ICA69-
13 deficient NOD mice to analyze the role of ICA69 in
14 autoimmunity¹⁷. These animals develop Type 1 diabetes with
15 slight delay at essentially wild type rates, assigning a
16 facultative rather than obligate role to ICA69 in diabetes
17 development¹⁷.

18 In accordance with the instant invention, it has been
19 determined that ICA69 deficient NOD females have dramatically
20 impaired development of pSS and its associated exocrinopathy.
21 Modification of T cell immunity to ICA69/Tep69 by
22 immunotherapy prevented disease development and reduced
23 established disease in wild type NOD mice. Extending these
24 observations to humans, we observed both T cell and

1 autoantibody responses to ICA69 in pSS patients, but not in
2 healthy controls or patients with SLE. The instantly
3 disclosed data establish ICA69 as a new pSS autoantigen which
4 appears to be critically involved in disease progression.

5

6 **Methodology**

7 **Human Subjects**

8 Blood samples were obtained from patients (n=15) with
9 primary SS or SLE at the Arthritis Center at Toronto Western
10 Hospital and from healthy, adult volunteers through ethics-
11 board approved consent (n=12). pSS patients were female, had
12 documented xerostomia and xerophthalmia and met San Diego
13 disease criteria. All were anti-Ro antibody positive, 6 had
14 anti-fodrin autoantibodies and all had minor salivary gland
15 biopsy focus scores of >5. Healthy controls (n=12) of similar
16 age and gender profile were recruited from staff. Fresh blood
17 was used for T cell studies. In immunoblotting experiments,
18 sera from patients and controls were diluted at 1:1000 and
19 blotted on nitrocellulose containing 1mg of recombinant ICA69
20 protein to detect the presence of anti-ICA69 antibodies.

21 **Mice.**

22 NOD/Lt ($H2-IA^{g7}$) mice were bred and maintained according
23 to approved protocols in our conventional unit (85% diabetes

1 incidence in females, 36 weeks of age). This study was based
2 on experiments with approximately 200 mice. The generation of
3 ICA69^{-/-} speed congenic NOD mice has been described¹⁷. In these
4 animals, all 17 *Idd* loci²⁰ were homozygous NOD as assessed
5 with microsatellite markers in the 5th backcross generation¹⁷.
6 Knockout animals in this report were derived from the 10th
7 backcross.

8 **Mouse Histology.**

9 Submandibular and lacrimal glands were removed and fixed
10 in 10% buffered formalin for at least 24 hr. Tissue sections
11 were stained with hematoxylin/eosin. For sialoadenitis
12 scoring, two blinded observers enumerated the number of
13 mononuclear foci at 3-5 different tissue depressions (100
14 mm/depression) in 2 full glands from each animal. The scores
15 from the different levels and the two observers were
16 averaged. A 'small' mononuclear focus had <75 inflammatory
17 cells/section (400X magnification). A large focus had >75
18 inflammatory cells. Dacryoadenitis was diagnosed if at least
19 one mononuclear focus was detected in one of two lacrimal
20 glands from each mouse. In ICA69^{+/+} and wild type NOD mice,
21 dacryoadenitis often consisted of large masses of lymphocytes
22 infiltrating into the acinar tissue. Such infiltrations were
23 absent in all ICA69^{-/-} animals analyzed.

1 **Proteins, Peptides and Immunotherapy.**

2 Human recombinant ICA69-b was purified as described¹⁴.
3 Grade V bovine serum albumin (BSA) and Ovalbumin (OVA) were
4 purchased (Sigma, St. Louis, MO). Peptides were purchased
5 HPLC purified (>95%) and confirmed by mass spectroscopy
6 (numbers indicate the N-terminal amino acid position): Tep69
7 (ICA69-p36), AFIKATGKKEDE; ABBOS (BSA-p150), FKADEKKFWGKYLYE.
8 In immunotherapy experiments, NOD female mice, 10 weeks of
9 age, were given a single intraperitoneal injection (100 ml)
10 of either 200 mg ABBOS peptide or PBS, both emulsified at a
11 1:1 ratio in incomplete Freund's adjuvant (IFA). Control mice
12 were untreated. Organs were harvested for histopathology at
13 various times after treatment.

14 **Proliferative T cell Responses.**

15 NOD lymph-node, spleen and human peripheral blood T cell
16 responses were measured with three slightly different
17 protocols. Draining lymph node cells from 10 week old NOD
18 females were pooled. 2×10^5 lymph node cells along with 2×10^5
19 irradiated (1100 rad) syngeneic spleen cells were cultured in
20 serum-free AIM-V media (Life Technologies, Mississauga,
21 Ontario, Canada) in the presence of protein or peptide
22 antigen. Proteins (ICA69, BSA or OVA) were used at
23 concentrations of 5 mg/ml, peptides (Tep69, ABBOS) at 50-100

1 mg/ml²¹. After 72 hr of incubation, cultures were pulsed
2 overnight with 1 mCi of [³H]thymidine, harvested and
3 subjected to liquid scintillation counting. Experiments were
4 repeated three times with similar results, each with lymph
5 nodes pooled from groups of 4-7 mice. Proliferation assays
6 with spleen cells used 4x10⁵ responding cells/well and no
7 irradiated splenocytes. For the detection of human T cell
8 responses, Ficoll-Hypaque purified peripheral blood
9 mononuclear cells (PBMC) were cultured at 10⁵ cells/well for
10 one week in serum-free Hybrimax 2897 medium (Sigma)
11 supplemented with human IL-2 (10 U/well) and 0.01-10 mg of
12 antigen¹⁵. This assay performed well in a large, blinded
13 study and in the first international T cell workshop of the
14 Immunology of Diabetes Society²².

15 **Statistics.**

16 Proliferative T cell responses were expressed as
17 stimulation index (SI, experimental/control cpm). SI's
18 greater than the mean SI in OVA-stimulated cultures plus 3 SD
19 were deemed positive¹⁵. Numeric data were compared by Mann-
20 Whitney tests, Fisher's exact test was used to analyze
21 tables. All *P* values were two-tailed and significance was set
22 at 5%. Figures present mean values plus 1SD.

23 **Protection from pSS in ICA69 deficient NOD congenic mice.**

1 The expression of ICA69 is similar in humans and
2 rodents¹⁸ and its presence in the submandibular glands of NOD
3 mice¹⁷ led us to examine the impact of ICA69 deficiency on
4 the development of NOD mouse sialoadenitis and
5 dacryoadenitis. Submandibular glands from NOD, ICA69^{+/−} and
6 ICA69^{−/−} NOD females of various ages were analyzed by two
7 blinded observers for the number and size of mononuclear cell
8 infiltration foci, values were within ±10%. Number and size
9 of mononuclear cell foci increased progressively with age in
10 heterozygous (ICA69^{+/−}) mice (Fig. 1A, B top panel). Timing
11 and progression of sialoadenitis in ICA69^{+/−} and wild type
12 mice was similar (*P* values >0.20, data not shown, equivalent
13 to Fig. 3A “untreated”), with initial infiltrates observed by
14 5–7 weeks of age. In striking contrast, sialoadenitis was
15 significantly reduced in ICA69^{−/−} NOD mice (Fig. 1A, B bottom
16 panel). Beginning usually around 9–10 weeks of age, ICA69
17 deficient animals developed mild salivary gland
18 infiltrations, that showed slow progression, on average 55–
19 65% below submandibular gland mononuclear foci observed in
20 wild type or heterozygote mice (*P* 0.006 vs. ICA69^{+/−} mice).
21 While ICA69 is not absolutely required for disease
22 initiation, its absence plays a lasting role during expansion
23 of the disease process, which shows little progression in

1 females older than 6 months of age. ICA69 therefore appears
2 to be involved in the progression of disease.

3 pSS in male NOD mice differs from the female phenotype,
4 with less sialoadenitis, but pronounced dacryoadenitis²³. The
5 cause of this gender bias is unclear, but unequal salivary
6 and lacrimal gland disease is common also in human pSS. Small
7 perivascular and periductal lymphocytic infiltrates of the
8 NOD male lacrimal gland appear around 10 weeks of age. By 30-
9 40 weeks of age, dacryoadenitis is conspicuous with extensive
10 lymphocyte infiltration into the acinar tissue and
11 progressive tissue destruction. In our colony, about two
12 thirds of wild type and 7/12 ICA69⁺⁻ NOD males between the
13 ages of 35-40 weeks exhibit definitive dacryoadenitis (Fig.
14 1C). In similarly aged male ICA69^{-/-} NOD mice, dacryoadenitis
15 was undetectable (0/12, Fig. 1C). Spontaneous autoimmune
16 inflammation of the lacrimal gland appears to require ICA69
17 expression.

18 **ICA69-specific T cell autoreactivity in the NOD mouse.**

19 These observations suggested a key role for ICA69
20 expression in the development and progression of NOD mouse
21 pSS. This phenotype could reflect a role for ICA69 as an
22 autoantigen or a role for ICA69 protein-function. To begin an
23 analysis of these two alternatives, we measured T cell
24 autoreactivity to ICA69 and its immunodominant T cell

1 epitope, Tep69, in 10 week old NOD females. Proliferative *in*
2 *vitro* recall responses were assessed in draining lymph nodes
3 from various tissues, in order to localize where T cell
4 tolerance to ICA69 was lost. Proliferative T cell responses
5 to ICA69 and Tep69 were detected in both pancreatic and
6 submandibular lymph node cells (Fig. 2A, B), but not in
7 popliteal or axillary lymph nodes (Fig. 2C, D). Equally
8 exclusive to pancreatic and submandibular lymph node cells,
9 we observed T cell proliferative responses to bovine serum
10 albumin (BSA) and its immunodominant epitope ABBOS, a peptide
11 that displays amino acid homology and antigenic mimicry with
12 Tep69¹⁴. Spleen cell responses to ICA69, Tep69, BSA and ABBOS
13 were present as previously described by us²¹ and others²⁴
14 (data not shown, but for example see Fig. 3F). The
15 localization of spontaneous ICA69 immune responsiveness to
16 the submandibular lymph nodes specifically links ICA69
17 autoimmunity with the salivary glands, and suggests that
18 ICA69 is a candidate autoantigen in NOD mouse pSS.

19 To test this conclusion and determine the role for ICA69
20 autoimmunity in the progression of NOD mouse pSS, we employed
21 an immunotherapy strategy¹⁴. Treatment of NOD mice with the
22 ABBOS mimicry peptide induces long lasting T cell tolerance
23 to Tep69 in most animals, due to the high MHC class II
24 affinity of ABBOS²¹. We examined the effects of ABBOS

1 peptide-induced Tep69-specific T cell tolerance on the
2 development and course of NOD mouse sialoadenitis. In order
3 to detect possible therapeutic effects of the peptide, we
4 injected 10 wk old wild type NOD females with established
5 disease. Five, 10 and 15 weeks after a single intraperitoneal
6 injection of 200 mg ABBOS emulsified in oil (incomplete
7 Freund's adjuvant, IFA), submandibular glands were examined
8 for the number of mononuclear foci (Fig. 3A). Control mice,
9 untreated or injected with emulsified vehicle only, showed
10 severe and progressive sialoadenitis at all time intervals
11 after treatment (Fig. 3A, B). ABBOS treatment produced
12 variable results, with predominant disease protection in two
13 thirds of animals ($P < 0.001$, Fig. 3A red circles, C, D). In a
14 third of protected mice, sialoadenitis was reduced to nearly
15 absent (Fig 3C, D). However, in contrast to disease
16 protection, we observed moderate disease exacerbation in a
17 subset of ABBOS treated mice (2/17 mice analyzed (12%), Fig
18 3A blue circles, E). Thus, a single injection of the
19 immunotherapeutic agent, ABBOS²¹, can affect progression and
20 induce regression of established NOD Sjögren's disease.
21 To analyze the variability of disease effects observed
22 following ABBOS-immunotherapy, we measured relevant splenic T
23 cell autoreactivity 5, 10 and 15 weeks following treatment
24 and compared the outcome with disease status. As expected^{14, 21},

1 mice treated with emulsified buffer (PBS-IFA, n=6) had T cell
2 recall responses to both, Tep69 and ABBOS peptides (Fig 3F).
3 Similarly, we observed Tep69 and ABBOS proliferative
4 responses in ABBOS treated animals that were not protected
5 from disease (Fig. 3F, n=6), including mice that displayed
6 moderate disease exacerbation. However, T cell responses to
7 Tep69 were greatly reduced in those mice that displayed
8 protection from sialoadenitis (n=11). Thus, ABBOS treatment
9 had selectively eliminated mimicry T cell pools that could
10 recognize the self-peptide, Tep69, inducing a bias for ABBOS
11 recognition only. The presence of ABBOS, but not Tep69 T cell
12 responses following ABBOS immunotherapy of the NOD mouse was
13 previously associated with diabetes prevention¹⁴, and likely
14 reflects selection of lower affinity T cell pools that cannot
15 be activated by Tep69 due to its very low MHC class II
16 affinity²¹. Taken together, these data indicate that
17 ICA69/Tep69 specific T cell pools are critical in sustaining
18 the natural progression of sialoadenitis in NOD mice, and
19 establish a driving role for ICA69 in the development of pSS.

20 **ICA69 Autoimmunity in primary SS patients**

21 To determine if ICA69 was an autoimmune target in
22 patients with primary SS, we first measured T cell responses
23 to ICA69 and Tep69 in PBMC from patients with primary SS
24 (n=9), systemic lupus erythematosus (SLE, n=6) and age-

1 matched healthy controls (n=12) (Fig. 7A). Positive responses
2 to both ICA69 and Tep69 were observed in 8 of 9 patients with
3 primary SS and were absent in patients with SLE and in
4 healthy controls ($P 0.008$ vs. SLE; $P 0.004$ vs. healthy
5 controls). These data identify T cell autoimmunity to ICA69
6 as a common characteristic of primary SS in humans. The
7 absence of ICA69/Tep69 specific T cell responses in SLE
8 patients suggests that autoimmunity to ICA69 may be used as a
9 marker to differentiate between the two diseases, which share
10 several autoimmune targets.

11 Immunoblotting was employed with patient sera to detect
12 the presence of autoantibodies against ICA69 (Fig. 7B).
13 Consistent with the presence of anti-ICA69 T cell autoimmunity,
14 sera from 8 of 9 pSS patients were positive for ICA69
15 antibodies. No immunoreactivity was observed in sera from SLE
16 patients (n=6) or healthy controls (n=12) (Fig. 7B). Our data
17 therefore establish ICA69 as an autoantigen in both NOD mouse
18 and human pSS. The generation of more patient data and family
19 studies are underway to determine the diagnostic significance
20 of anti-ICA69 immunoreactivity in this disease.

21 In conclusion, the instant invention evidences a
22 dramatic protection from pSS in ICA69-deficient NOD mice. The
23 reduction of sialoadenitis in ICA69^{-/-} mice is most likely the
24 result of absent ICA69-specific autoimmunity. This conclusion

1 is supported by the presence of ICA69-specific T cell
2 responses in submandibular lymph nodes and spleens of wild
3 type NOD mice and in peripheral blood of patients with
4 primary SS. These T cell proliferative responses and in
5 particular the tight correlations between ICA69-specific
6 autoimmunity and disease status during peptide-based
7 immunotherapy further emphasizes the link between pSS and
8 ICA69. While we can not completely rule out a role for
9 functional properties of ICA69 in disease development, as the
10 function of the molecule remains unclear, nevertheless, the
11 identification of ICA69 as a new autoantigen in pSS may
12 provide a new marker for disease diagnosis and a new target
13 for disease preventive therapy.

14 It has been previously observed that NOD tolerance
15 induction and disease protection by ABBOS are dose dependent
16 peptide effects, with failure of tolerization and disease
17 acceleration/precipitation at suboptimal peptide doses²¹. The
18 observed variation of ABBOS effects on tolerization and pSS
19 disease progression likely reflects variances in the rate of
20 peptide release from the oily emulsion applied and/or subtle
21 differences in T cell repertoires. There was no quantitative
22 relationship between the extent of tissue lesions and T- or B
23 cell autoimmunity in established pSS of patients and NOD
24 mice, suggesting that these autoreactivities reflect more the

1 presence than the extent of tissue damage. However, following
2 immunotherapy, tissue infiltration and autoreactivity changed
3 closely in parallel. Immunotherapy-induced changes in ICA69
4 autoimmune status may provide a read-out of effectiveness.

5 The search for autoantigens in pSS identified several
6 members of nuclear complexes (e.g. SS-A/Ro, SS-B/La, and SS-
7 56), as well as more tissue-specific antigens such as a-
8 fodrin, b-fodrin, and the muscarinic M3 receptor¹². In
9 addition to the submandibular and lacrimal glands, ICA69 is
10 also expressed in pancreatic beta cells and nervous system
11 tissue. A high incidence of up to 40% of pSS patients
12 manifest neurological complications, often with
13 polyneuropathy and the appearance of anti-neuronal
14 autoantibodies²⁵⁻²⁷. It is conceivable that autoimmune
15 targeting of ICA69 may play a role in spreading of autoimmune
16 disease to nervous system tissue. This cytosolic molecule is
17 a prominent target in human and NOD mouse pSS, type 1
18 diabetes and in MS, where different epitopes are targeted¹⁹.
19 Studies are under way to determine if the shared targeting of
20 Tep69/ABBOS is related to the high prevalence of DR3 in pSS,
21 which is shared with diabetes^{28,29}.

22 T cells are believed to drive the histopathological
23 changes in pSS, yet the significance of T cell targeting of
24 autoantigens identified previously is not known³⁰. However,

1 immunity to a-fodrin, was shown to be critical for the
2 development of salivary and lacrimal gland exocrinopathy in
3 NFS/sld mice⁶. Mild sialoadenitis does develop in ICA69
4 deficient mice, but with a considerable decrease in rate of
5 progression and severity. These observations suggest that T
6 cell targeting of ICA69 may be more central to the
7 progression phase of disease after it has been initiated,
8 possibly through autoimmune targeting of other autoantigens
9 such as a-fodrin³¹. A hierarchy of autoantigen targeting and
10 antigen spreading from few to many has been proposed in
11 several autoimmune conditions.

12 Autoimmunity to ICA69 appears to be essential for the
13 development of NOD mouse dacryoadenitis, a disease related
14 to, but distinct from sialoadenitis by several criteria.
15 ICA69^{-/-} NOD males as old as one year failed to develop
16 histological signs of dacryoadenitis. Differences in disease
17 development between lacrimal and salivary gland infiltration
18 are common. pSS patients can develop sialoadenitis with or
19 without dacryoadenitis and vice versa¹. A requirement for
20 autoimmune targeting of ICA69 in the manifestation of
21 dacryoadenitis identifies ICA69 as a critical antigen in the
22 initiation of this disease. It will be interesting to
23 determine if the pSS-like disease of other mouse strains,
24 such as MRL/lpr, also involves autoimmune targeting of ICA69.

1 What elements may contribute to the loss of tolerance to
2 ICA69 and subsequent priming of T and B cells? One factor
3 may lie in the extensive remodeling and apoptosis observed in
4 the salivary glands of NOD and immunodeficient NOD.scid
5 mice^{32,33}. This process could liberate ICA69 antigen to
6 draining lymph nodes or antigen presenting cells found in the
7 tissue, subsequently resulting in T cell activation.
8 Consistently, we observed spontaneous ICA69/Tep69-specific T
9 cell responses in draining submandibular lymph nodes of 10
10 week old NOD females. General defects in the immune system
11 likely contribute to disease. For example, elevated levels of
12 the TNF superfamily member, B cell activating factor (BAFF),
13 have been observed in pSS patients, and transgenic expression
14 of BAFF produces pSS in C57BL/6 mice³⁴. Such abnormalities
15 may promote systemic defects in self-tolerance, which may
16 include prominent autoimmunity to ICA69.

17 Because of the diversity and variability of human pSS,
18 translation of data from the NOD mouse to human disease must
19 be met with caution. However, the identification of ICA69 as
20 a novel and perhaps central autoantigen in pSS has
21 ramifications. Antibodies to ICA69 could be used as markers
22 in disease diagnosis and to serologically differentiate
23 between pSS and SLE. In addition, non-toxic immunotherapies
24 aimed at depleting ICA69/Tep69-reactive T cell pools could be

1 a candidate therapy to halt and reverse disease progression.

2 In U.S. Patent 6,207,389, the contents of which is
3 incorporated herein in its entirety, we have previously
4 associated the efficiency of ABBOS immunotherapy in diabetes
5 prevention with its high affinity binding to MHC, where the
6 tolerogenic effect of ABBOS was dose-dependent, and
7 predictable disease exacerbation was observed at suboptimal
8 doses²¹. This raises caution in the translation of mouse to
9 human data, in particular with the choice of peptide and
10 peptide doses. Thus, ABBOS homologs with even higher affinity
11 should be considered for optimal and safer immunotherapy,
12 which could be monitored with biopsies and T cell assays.

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4 The above references were relied upon and are
5 incorporated by reference herein in their entirety.

6 All patents and publications mentioned in this
7 specification are indicative of the levels of those skilled
8 in the art to which the invention pertains. All patents and
9 publications are herein incorporated by reference to the same
10 extent as if each individual publication was specifically and
11 individually indicated to be incorporated by reference.

12 It is to be understood that while a certain form of the invention is illustrated, it is not to be limited to the specific form or arrangement herein described and shown. It will be apparent to those skilled in the art that various changes may be made without departing from the scope of the invention and the invention is not to be considered limited to what is shown and described in the specification.

19 One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objectives and obtain the ends and advantages mentioned, as well as those inherent therein. The embodiments, methods, procedures and techniques described herein are presently representative of the preferred embodiments, are intended to be exemplary

1 and are not intended as limitations on the scope. Changes
2 therein and other uses will occur to those skilled in the art
3 which are encompassed within the spirit of the invention and
4 are defined by the scope of the appended claims. Although
5 the invention has been described in connection with specific
6 preferred embodiments, it should be understood that the
7 invention as claimed should not be unduly limited to such
8 specific embodiments. Indeed, various modifications of the
9 described modes for carrying out the invention which are
10 obvious to those skilled in the art are intended to be within
11 the scope of the following claims.

12